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Separation of Fungal Carbohydrases by Starch Block Zone Electrophoresis

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Several carbohydrases found in culture filtrates of various fungi show multiple components when resolved by starch block zone electrophoresis. Patterns obtained from carbohydrases from different fungi vary considerably.

Different carbohydrases from a single fungus usually migrate at different rates so that the activities can be separated from each other by electrophoresis. However, individual electrophoretic components may reveal enzymic activities to varying degrees on more than one substrate.

Possible explanations for the multiple nature of carbohydrases are discussed.

INTRODUCTION

The use of starch block zone electrophoresis in studies on cellulase (1, 2) suggested its use for other carbohydrases. It was of interest to determine whether other carbohydrases would, like cellulase, show multiple components, and whether individual carbohydrases might act on more than one type of carbohydrate. Filtrates of fungus cultures were particularly suitable for such studies since they may contain variable quantities and numbers of carbohydrases, depending upon the nature of the fungus and the cultural conditions (3).

METHODS

Enzymes

The enzyme preparations used (Table I) were cell-free filtrates of fungus cultures, used either directly or after precipitation with acetone, drying, and redissolving in water. Before being placed on the starch block, the enzyme solutions were dialyzed in animal membranes (Aronab Products Co., San Francisco, Calif.) against distilled water, concentrated several fold by means of a rotating vacuum evaporator, and buffered with phosphate to give a final pH of 7 and ionic strength 0.1.

Electrophoresis

The starch block zone electrophoresis was carried out in the apparatus described by Miller (4), using a trough of dimensions 100 × 3.3 × 0.3 cm. One-tenth-milliliter samples of enzyme solutions, to which 5-mg. quantities of dextran were added as indexes of the starting positions, were introduced into transverse slits in the centers of the blocks. The quantities of enzyme present in the samples varied considerably, as indicated in Table I. However, it was found in separate studies, not shown, that sizes of samples affected electrophoretic separations to a relatively minor degree. To minimize the possibility that rapidly migrating enzyme components might migrate off the ends of the blocks, the runs were carried out at 4° and 8 v./cm. for only 20 hr. instead of for the 40-hr. period previously used (4). Also, it was not considered necessary for purposes of the present study to cut the blocks into pieces smaller than 1 cm. at the ends of the runs.

Enzyme Activity

Determinations of distributions of enzyme activities in the starch blocks were based on the production of reducing groups by the hydrolysis of glycosidic bonds and were carried out using modifications of previously published methods (5–7). After the electrophoresis, the blocks were cut into 1-cm. pieces and each piece was placed in a test

TABLE I

ENZYME PREPARATIONS USED IN THIS STUDY

Organism	QM No.	Cultural conditions	Enzymes (units on starch block) Glucanases		
			β-(1 → 4)		β-(1 → 3)
Penicillium pusillum	137g	Unshaken, duck strip	100		20
Trichoderma viride	6a	Shake culture, Solka floc[a]	33		17
Pestalotiopsis westerdijkii	381	Shake culture, Solka floc	28		4.4
Basidiomycete	806	Shake culture, Solka floc	70		33
Sporotrichum pruinosum	826	Shake culture, Solka floc	27		13
Streptomyces sp.	B814	Shake culture, Solka floc	8.2		0.5
Myrothecium verrucaria[b]	460	Shake, ground filter paper	100		11
			Fructanases		
			Sucrase	β-(2→1)	β-(2→6)
Penicillium funiculosum	474	Unshaken, inulin	114	11	2
Fusarium moniliforme	527	Unshaken, inulin	1.5	—	1

[a] Purified wood cellulose, Brown Co., Berlin, N. H.

[b] Enzyme preparation used by Miller *et al.* (1, 2).

tube containing 8 ml. water. The contents of the tubes were mixed well, and the starch was allowed to settle out overnight. Enzyme determinations were then made by adding 0.5–1.0 ml. of a suitable dilution of the supernatant to an equal volume of the appropriate substrate made up in 0.1 *M* citrate buffer, generally at pH 5.3. Glucose was usually added to the substrate to give 0.1 mg. per sample to compensate for the glucose destroyed by the color reagent used (8). The mixtures of enzyme and substrate were incubated at 50°, 3 ml. dinitrosalicylic acid reagent (8) was added to each, and the tubes were heated for 15 min. at 100°. The color produced was measured in an automatic recording colorimeter which plotted the values directly as absorbance (9). Wide ranges in enzyme activity were handled by variation in quantity of enzyme, time of incubation, and wavelength of absorbance.

Cx (cellulase) activity was measured against 0.5% final concentration of carboxymethylcellulose (Hercules CMC-50-T). Activity on solid cellulose was measured against a 0.5% suspension of ground cotton linters swollen with phosphoric acid after the procedure of Walseth (10); undigested cellulose was removed before determination of the sugar produced. The substrate used for β-(1 → 3) glucanase was 0.3% laminarin (Institute of Seaweed Research, Inveresk, Scotland); the pH, 4.8. Sucrase was measured against 0.25% sucrose (Merck). The substrate for β-(2 → 1) fructanase was 0.15% inulin (Difco). The substrate for β-(2 → 6) fructanase was a 0.15% solution of the levan produced by *Acetobacter acetigenum* (7). The unit of activity for cellulase has been described previously (5). For the other enzymes, the unit of activity was that amount of enzyme producing 1.0 mg. sugar as glucose per 2 ml. per hour in the tests.

By reference to a set of standards, composed of appropriate dilutions of the original enzyme concentrates, recoveries of enzyme activities for each set of unknown electrophoretic fractions could be calculated. For presentation of data showing distributions of enzyme components, however, enzyme activities are given simply in terms of absorbance of the colored solutions obtained in the determinations of reducing sugar produced since the relationships between amounts of enzyme and absorbance were fairly linear over the ranges used. Further, because of the variations in the initial quantities of enzyme in the different tests and in the wavelengths at which absorbances were measured, absorbances are expressed in arbitrary units which are not given numerical values. For any given enzyme the relative quantities of different components are indicated to a first approximation by the sizes of the peaks. However, exact quantitative significance cannot generally be attached to sizes of peaks when comparisons are made between results obtained in different experiments or with different enzymes.

The position of the starting zone on the starch blocks, which moved slightly because of electroendosmosis, was found by locating the dextran with the orcinol reagent of Rimington (11).

Paper Chromatography

Electrophoretic fractions forming a single peak of activity were combined and concentrated in a rotating vacuum evaporator. Five-tenths milliliter of concentrate was added to 0.3 ml. of 2% substrate in 0.05 *M* citrate buffer at pH 5.0 and incubated for 4 hr. at 50°. A comparable dilution of the original unfractionated enzyme was included in the test. Digests and known sugars were spotted on sheets of Whatman No. 1 filter paper and were developed for 16 hr. with isopropyl alcohol–glacial acetic acid–water 54:8:18. Reducing sugars were located by spraying the sheets with a solution composed of 0.5 g. benzidine, 10 ml. of glacial acetic acid, 10 ml. of 40% trichloroacetic acid, and 80 ml. ethanol.

RESULTS

Zone Electrophoretic Studies

Zone electrophoretic patterns for filtrates from six different fungi, each containing mixtures of β-(1 → 4) glucanase (cellulase) and β-(1 → 3) glucanase (laminarinase), are shown in Fig. 1. The portions of the blocks for which data are shown include all of the active components which were observed. In each case the enzymes were resolved into several zones, or at least showed a spread of zone, which signified the occurrence of multiple electrophoretic components. This finding was substantiated by the results of additional runs, not shown, in which the duration of electrophoresis was increased and the sizes of pieces into which the starch blocks were cut were decreased. Furthermore, enzymes having the same specificity but produced by different fungi can have different rates of migration. Of greater interest, perhaps, is the observation that in most cases components within an individual filtrate which have different specificities also have distinctly different rates of migration. This offers a means of preparing a single enzyme free of other activities.

In relatively fewer instances components having different specificities have the same rates of migration. This may, or may not, be coincidental. Further information on this question is provided by the results of electrophoretic studies on filtrates containing various mixtures of β-(2 → 6) fructanase, β-(2 → 1) fructanase, and sucrase, shown in Fig. 2. In these systems it appears that individual electrophoretic components may act upon more than one substrate. For example, in the filtrate from *Fusarium moniliforme*, an electrophoretic component acting on β-(2 → 6) fructan also acts on sucrose, although other components which act on sucrose cannot act on β-(2 → 6) fructan. In the filtrate from *Penicillium funiculosum*, components acting on β-(2 → 6) and those acting on β-(2 → 1) fructan all act on sucrose. Further inspection of the patterns reveals also that electrophoretic components showing strong activity on β-(2 → 1) fructan show slight activity on β-(2 → 6) fructan.

Recoveries of enzyme activities usually approached 100% but occasionally fell as low as 50% (*Trichoderma viride* on solid cellulose) or went as high as 140% (*Myrothecium verrucaria* on laminarin). The reasons for low or high recoveries were not investigated. The low recovery for *T. viride* on solid cellulose may be a reflection of the synergistic action found by Gilligan and Reese (5).

Chromatographic Studies

Paper chromatographic patterns for enzymic digests resulting from the action on β-(1 → 3) glucan (laminarin) of two electrophoretically distinct β-(1 → 3) glucanase fractions and one β-(1 → 4) glucanase fraction from *Penicillium pusillum* (Fig. 1) are presented in Fig. 3. The results show that the slowly migrating β-(1 → 3) glucanase produced predominantly glucose while the rapidly migrating β-(1 → 3) glucanase produced predominantly laminaribiose and laminaritriose. Thus the former is an exo- or endwise splitting enzyme, while the latter is an endo- or random splitting enzyme (12). When the original enzyme was used, glucose was the only product found and the presence of the random splitting component was not apparent. The occurrence of these two types of β-(1 → 3) glucanases has been demonstrated previously but in filtrates of different organisms (6). It can be seen also in Fig. 3 that the β-(1 → 4) glucanase had essentially no action on the β-(1 → 3) glucan.

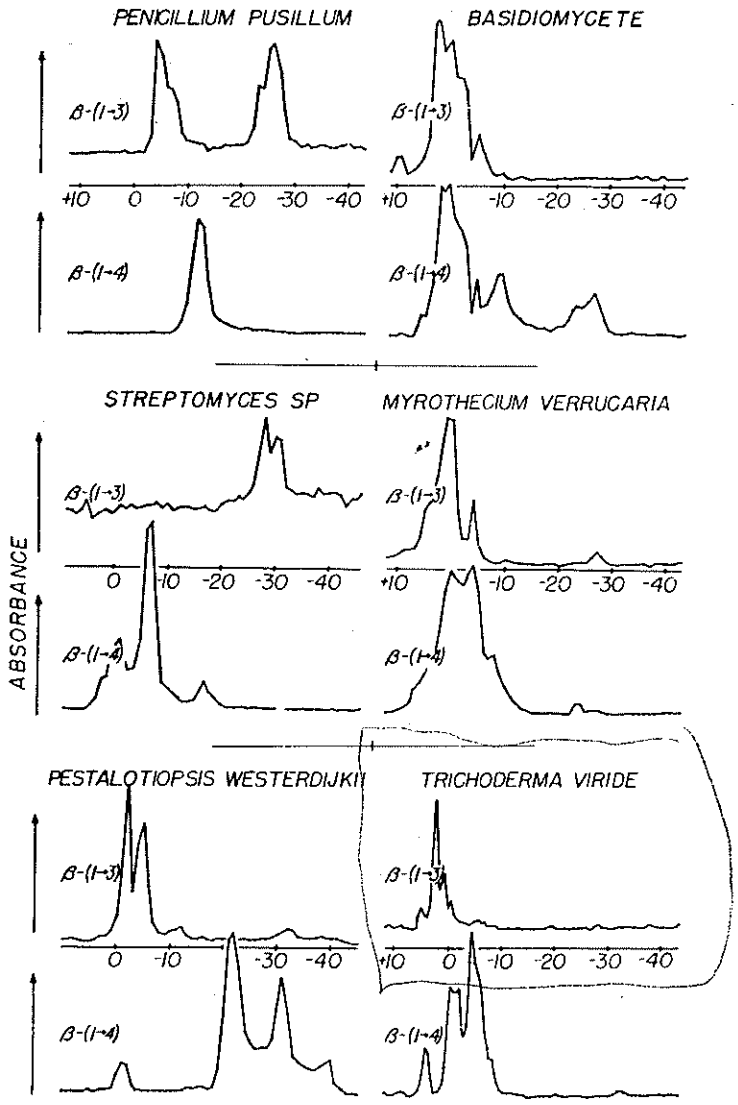

FIG. 1. Separation of β-(1 → 3) and β-(1 → 4) glucanases by zone electrophoresis.

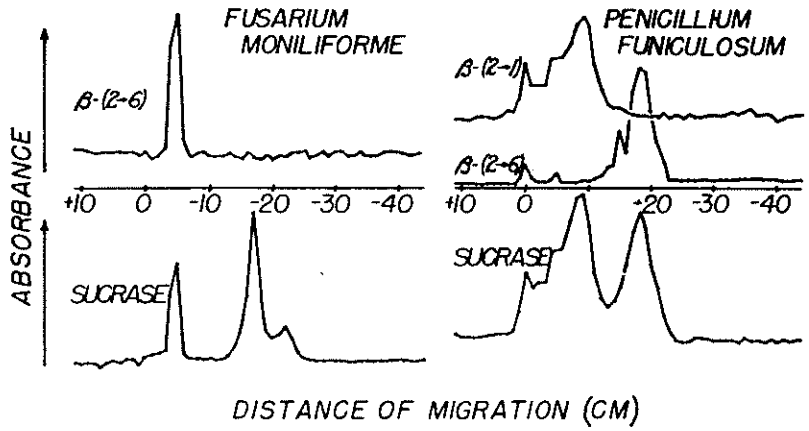

FIG. 2. Separation of β-(2 → 1) fructanase, β-(2 → 6) fructanase and sucrase by zone electrophoresis.

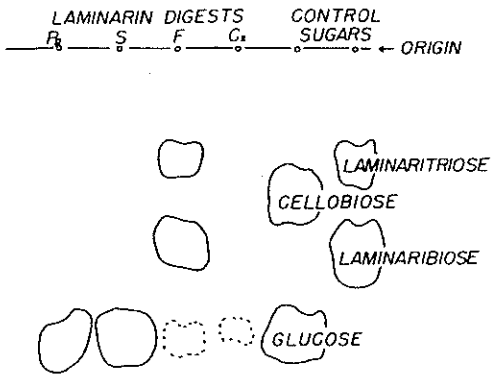

FIG. 3. Products formed when a culture filtrate of *Penicillium pusillum* and certain electrophoretic components of this filtrate acted on 0.75% laminarin. P.p. dilution of original filtrate 6 Cx units, 1.2 β-(1 → 3) glucanase units/ml. digest. S. slow β-(1 → 3) glucanase component (−4 to −7 cm., Fig. 1). 0.9 β-(1 → 3) glucanase units/ml. digest. F. fast β-(1 → 3) glucanase component (−23 to −27 cm., Fig. 1). 1.2 β-(1 → 3) glucanase units/ml. digest. Cx. Cellulase component (−10 to −14 cm., Fig. 1) 2 Cx units/ml. digest.

A similar analysis of products of selected electrophoretic fractions of β-(1 → 3) glucanases of *T. viride, Pestalotiopsis westerdijkii, Streptomyces* sp., *Aspergillus luchuensis* QM 473, *P. funiculosum* QM 474, and *Rhizopus arrhizus* QM 1032 acting on laminarin and the β-(1 → 4) glucanases of *T. viride, P. westerdijkii,* and *Streptomyces* sp. acting on solid cellulose was made. In these instances no clear-cut differences in mode of action by different components of a single enzyme were found.

Comparison of Solid Cellulose with Carboxymethylcellulose as Substrate for β-(1 → 4) Glucanase

Distribution patterns for β-(1 → 4) glucanase where solid cellulose was used as substrate were generally very similar to those obtained with carboxymethylcellulose, as shown by examples presented in Fig. 4. It can be concluded, therefore, that "cellulase" is being measured in either case. In

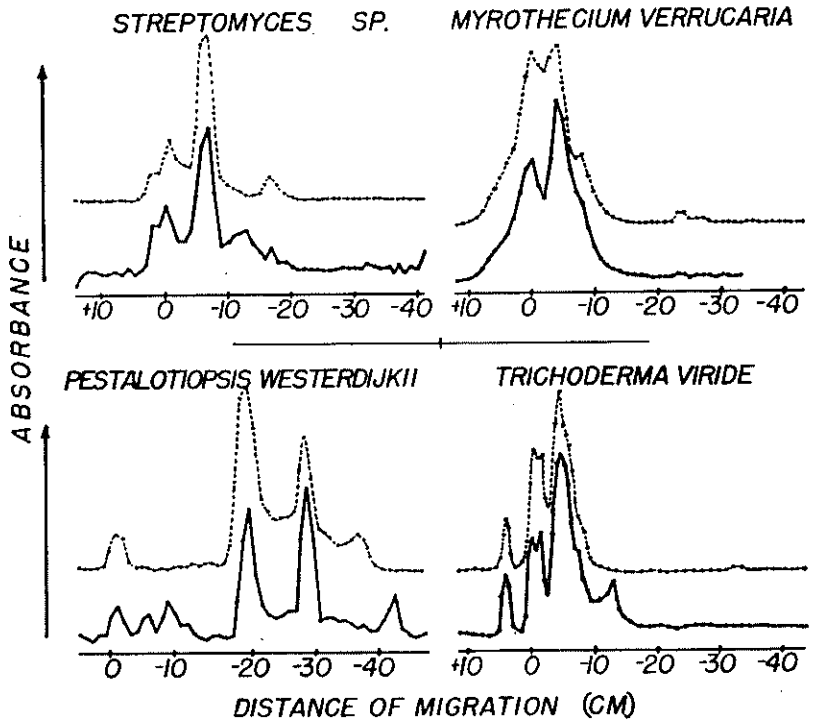

FIG. 4. Comparison of cellulase action on carboxymethylcellulose with that on solid (Walseth) cellulose. ----Cx, —— Walseth.

certain tests, however, it will be noted that minor electrophoretic components occurred which were active only on solid cellulose or on carboxymethylcellulose.

DISCUSSION

The multiple nature already demonstrated for a number of carbohydrases (2) receives further support from the present study, and indeed it begins to appear that all carbohydrases may be multiple. The members of such multiple systems may be referred to as isozymes, a term suggested by Markert and Møller (13).

The problem now is to find explanations for the occurrence of isozymes in carbohydrases. One explanation is provided, at least partially, by the demonstration in this work that different isozymes may function enzymically by different mechanisms. This is not likely to be the sole explanation, however, since we have found it to apply to only one of several preparations examined and Storvick and King (14) have reported that four cellulolytic components from *Cellvibrio gilvus* all function by the same mechanism. Another possible explanation in the case of polysaccharases may be that different isozymes are specific for different chain lengths of substrate, some evidence for which has been presented by Reese and co-workers (5, 15) and by Hash and King (16).

Still another explanation might be the existence of a number of basic protein skeletons, any of which may acquire enzyme specificity by the presence of particular amino acid sequences within limited portions of the molecule. The existence of a large number of protein "carriers" in a single fungus culture may reflect the existence of a number of synthetic pathways, genetic inhomogeneity, variations in age and nutrition of the cells producing the proteins, or proteolytic or denaturing influences which affect protein structure, after secretion, without affecting enzyme activity. Thomas and Whitaker (17), adhering to a uni-enzymic theory of cellulase activity, suggest that a single enzyme component may achieve multiplicity by complexing with polysaccharides or other products occurring in the environment.

In mosts cases β-(1 $\rightarrow$ 3) and β-(1 $\rightarrow$ 4) glucanase components from a single preparation showed different rates of migration indicating that these activities are properties of separate and distinct enzymes. The existence of overlapping components and of peaks showing a major activity on one substrate and a minor activity on a second substrate may indicate that we have not yet succeeded in completely separating different activities. On the other hand, Pazur and Ando (18) report the purification of a single enzyme hydrolyzing α-(1 $\rightarrow$ 4) and α-(1 $\rightarrow$ 6) glucosidic linkages. If, indeed, a single protein may show activity on different substrates, then the assumption of a graded series of enzymes showing different ratios of activities on different substrates would allow the existence of a limitless number of components.

Zone electrophoresis appears to be a practical means for separating carbohydrase components of different specificities from one another. The different highly purified fractions which are obtained may be of value in establishing proof of structure of unknown carbohydrates or in specifically removing components from mixtures of carbohydrases.

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REFERENCES

1. Miller, G. L., and Blum, R., *J. Biol. Chem.* **218,** 131 (1956).
2. Miller, G. L., Blum, R., and Hamilton, N., *J. Chromatog.* **3,** 576 (1960).
3. Reese, E. T., and Mandels, M., *Appl. Microbiol.* **7,** 378 (1959).
4. Miller, G. L., *J. Chromatog.* **3,** 570 (1960).
5. Gilligan, W., and Reese, E. T., *Can. J. Microbiol.* **1,** 90 (1954).
6. Reese, E. T., and Mandels, M., *Can. J. Microbiol.* **5,** 173 (1959).
7. Loewenberg, J., and Reese, E. T., *Can. J. Microbiol.* **3,** 643 (1957).
8. Miller, G. L., *Anal. Chem.* **31,** 426 (1959).
9. Miller, G. L., and Cummings, C., *Anal. Chem.* **31,** 481 (1959).

10. WALSETH, C. S., *Tappi* 228 (1952).
11. RIMINGTON, C., *Biochem. J.* **34**, 931 (1940).
12. DUNCAN, W. A. M., MANNERS, D. J., AND ROSS, A. G., *Biochem. J.* **63**, 44 (1956).
13. MARKERT, C. L., AND MØLLER, F., *Proc. Natl. Acad. Sci. U. S.* **45**, 753 (1959).
14. STORVICK, W. O., AND KING, K., *J. Biol. Chem.* **235**, 303 (1959).
15. REESE, E. T., SMAKULA, E., AND PERLIN, A. S., *Arch. Biochem. Biophys.* **85**, 171 (1959).
16. HASH, J. H., AND KING, K., *J. Biol. Chem.* **232**, 381 (1958).
17. THOMAS, R., AND WHITAKER, D. R., *Nature* **181**, 715 (1958).
18. PAZUR, J., AND ANDO, T., *J. Biol. Chem.* **235**, 297 (1960).